

Macromolecular crystal growth experiments on International Microgravity Laboratory—1

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Abstract

Macromolecular crystal growth experiments, using satellite tobacco mosaic virus (STMV) and canavalin from jack beans as samples, were conducted on a US Space Shuttle mission designated International Microgravity Laboratory—1 (IML-1), flown January 22–29, 1992. Parallel experiments using identical samples were carried out in both a vapor diffusion-based device (PCG) and a liquid-liquid diffusion-based instrument (CRYOSTAT). The experiments in each device were run at 20–22 °C and at colder temperatures.

Crystals were grown in virtually every trial, but the characteristics of the crystals were highly dependent on the crystallization technique employed and the temperature experience of the sample. In general, very good results, based on visual inspection of the crystals, were obtained in both PCG and CRYOSTAT. Unusually impressive results were, however, achieved for STMV in the CRYOSTAT instrument. STMV crystals grown in microgravity by liquid-liquid diffusion were more than 10-fold greater in total volume than any STMV crystals previously grown in the laboratory.

X-ray diffraction data collected from eight STMV crystals grown in CRYOSTAT demonstrated a substantial improvement in diffraction quality over the entire resolution range when compared to data from crystals grown on Earth. In addition, the extent of the diffraction pattern for the STMV crystals grown in space extended to 1.8 Å resolution, whereas the best crystals that were ever grown under conditions of Earth's gravity produced data limited to 2.3 Å resolution.

Other observations indicate that the growth of macromolecular crystals is indeed influenced by the presence or absence of gravity. These observations further suggest, consistent with earlier results, that the elimination of gravity provides a more favorable environment for such processes.

Keywords: canavalin; crystallization; microgravity; protein crystals; STMV; X-ray crystallography

There are three factors influencing macromolecular crystal growth that might be affected by the reduction or elimination of gravity. First, no containing vessel need be used in zero gravity because a sample of mother liquor, of nearly any volume, can be freely suspended in space. Thus the mother liquor need be bounded only by a solvent-air interface and would not be influenced by contact with any foreign surface. This may be relevant because surface effects most certainly affect the nucleation of crystals and their subsequent growth (McPherson & Shlichta, 1988).

Second, crystals grown from aqueous media invariably sediment, particularly when they grow large. Consequently, they come into contact either with a container

surface and/or with other growing crystals. Again, a crystal experiencing the influence of surface contact will frequently show decreased perfection. In addition, sedimentation due to gravity both disturbs the local environment of growing crystals and limits the technical approaches we might otherwise consider for their growth (Koszelak & McPherson, 1988; Koszelak et al., 1991).

Finally, elimination of gravity removes the effect of density-driven convective flow. Such flows produce turbulence at the faces of growing crystals (Pusey & Naumann, 1986) and increase the occurrence of dislocations and other imperfections. In addition, convective mixing, taking place in the presence of gravity, prevents the formation of stable depletion zones around growing crystals, which provide more ordered and regulated addition of macromolecules to growing crystal surfaces (McPherson et al., 1991). This is particularly important because mac-

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romolecular crystals are grown at very high levels of supersaturation, often reaching several hundred percent.

Evidence has accumulated over the past several years, from a variety of experiments carried out in microgravity, that protein crystals of improved qualities or increased order can be grown under reduced gravity conditions (Littke and John, 1984, 1986; Littke, 1985, 1988; DeLucas et al., 1986, 1989, 1991a,b; DeLucas & Bugg, 1987; Giege et al., 1988; Erdmann et al., 1989; McPherson et al., 1991). Because of the infrequency of flight opportunities and the restricted selection of experimental techniques available to the individual investigator, reproducibility, optimization, and comparison of results is difficult. For most investigators interested in the problem of protein crystal growth in space, the crystal growth apparatus currently available is the vapor diffusion apparatus (VDA), which is flown by NASA under the experiment title PCG (for Protein Crystal Growth). This system was designed and constructed under the guidance of Dr. Charles Bugg at the University of Alabama at Birmingham and is generally made freely available to interested investigators (DeLucas et al., 1986, 1989, 1991a,b).

Disadvantages of the PCG apparatus are that it provides only a single method for protein crystal growth, vapor diffusion in a hanging drop (McPherson, 1976, 1982, 1990, 1991), and usually only 5–10 samples of a particular protein can be carried into space on a Space Shuttle mission. Nonetheless, experiments in this device have provided the majority of results for several proteins, and this has proven to be the pioneer for the field (DeLucas et al., 1989; McPherson, 1991).

The Space Shuttle mission designated International Microgravity Laboratory—1 (IML-1), flown January 22–29, 1992, was wholly dedicated to microgravity research in space and carried, in addition to the PCG apparatus, a second device also designed for the crystallization of macromolecules. This instrument, termed CRYOSTAT, for its emphasis on accurate temperature control, employed a second technique for the growth of protein crystals, that known as liquid–liquid diffusion, or free interface diffusion (Weber & Goodkin, 1970; Salemm, 1971, 1972; McPherson, 1976, 1982, 1990, 1991).

PCG depends on the slow dehydration of a microdroplet of protein solution by equilibration with a precipitant containing reservoir, through the vapor phase. An example is shown in Figure 1. Liquid–liquid diffusion relies on the direct mixing of one liquid with another by direct diffusion across a free interface. It is essentially dialysis without recourse to a membrane. In the laboratory, direct liquid–liquid diffusion presents several problems for efficient protein crystal growth. In Earth's gravity, turbulent mixing at liquid–liquid interfaces, due to density-driven convection, results in rapid and unacceptable perturbations. Superimposed upon this is the effect of sedimentation, due to gravity, of any crystals that do form.

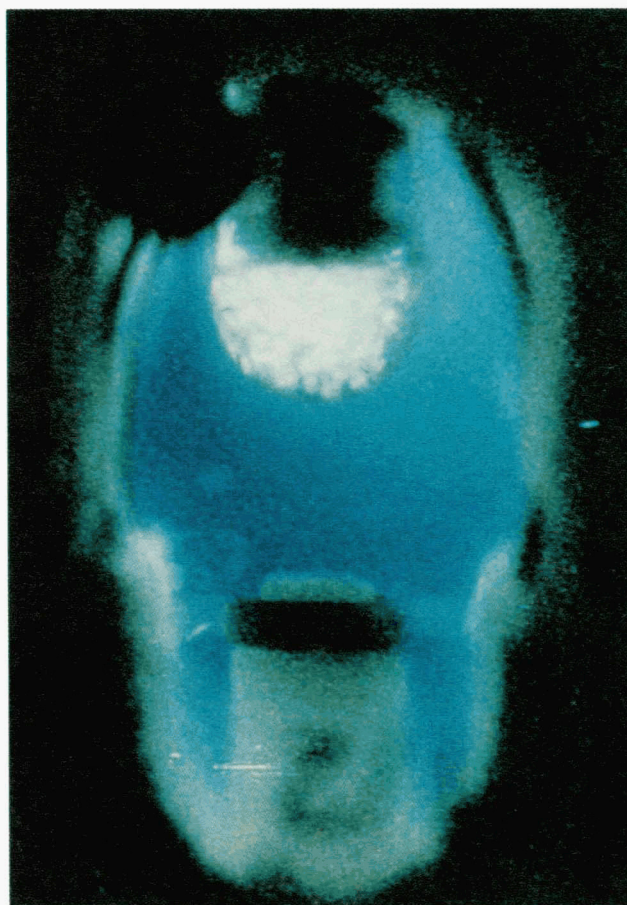


Fig. 1. Photograph taken in space (G. Nelson, astronaut, mission STS-61C, January 1986) of a hanging drop in the PCG device about 3 days after achievement of orbit. The 40- μ L droplet suspended from the bottom of its carrier syringe at the top of the chamber has canavalin crystals, seen in white, dispersed throughout its volume. The object below is a retaining plug that is moved to seal the syringe aperture during launch and reentry. Photograph courtesy of NASA.

Problems inherent to the liquid–liquid technique are, however, alleviated in microgravity. Both density-driven convection and sedimentation effects are eliminated as is the necessity for a membrane to separate protein from precipitant solution. Because of the extremely slow diffusion rates of macromolecules in comparison with small molecules, the protein component of the system remains essentially stationary during the entire course of the experiment. The precipitant diffuses into it thereby changing the protein's environment in a slow and precise manner.

On IML-1 we had the opportunity to fly two different samples of macromolecules, a protein and a virus, in parallel experiments using both the vapor diffusion-based device, PCG, and the CRYOSTAT. In addition, within each apparatus we were able to conduct experiments under two different sets of temperature conditions.

The protein sample was canavalin, the major storage protein from the jack bean (McPherson & Spencer, 1975;

Smith et al., 1982) that we have flown on previous Space Shuttle missions. It is reliable in its crystallization characteristics. The virus we utilized was satellite tobacco mosaic virus (STMV) a very small icosahedral virus (Valverde & Dodds, 1986, 1987; Mirkov et al., 1989; Dodds, 1991; Valverde et al., 1991) having a total weight of two million Daltons and whose structure has now been solved in this laboratory (Koszelak et al., 1989; Larson et al., 1992). Although the virus was used in some earlier experiments in the PCG, our experience with this sample was considerably less than for canavalin.

For both of our samples, extensive X-ray diffraction data were available from crystals grown on Earth. Examples of hexagonal canavalin and orthorhombic STMV crystals grown in our laboratory are shown in Figures 2 and 3, respectively. In addition, we have worked with the crystallization of both samples for 20 years in the case of canavalin (McPherson & Rich, 1973) and 5 years for STMV (Koszelak et al., 1989), and the characteristics and eccentricities of the crystals were reasonably well known to us.

Our fundamental objectives in these experiments were to obtain crystals of the protein and the virus in microgravity, to determine if the method of growth had a profound effect on the outcome, to evaluate the effects of temperature on the growth of these crystals in space, to observe any anomalies or unusual occurrences that might indicate a gravity effect, and, most important, to seek evidence that gravity is a significant factor in the growth of macromolecular crystals. Our results provided us with a variety of unique observations, a host of different crystal forms, and, most important, evidence that the phe-



Fig. 2. Typical hexagonal prismatic canavalin crystals grown in the laboratory on Earth at 4 °C. Note the distinctive, conical cusp that characterizes the growing end of the crystals. Rapid growth, as occurred here, frequently produces nutrient starvation at the center of the 001 face, which results in these cavities. Crystals seen here under polarized light are about 0.4 mm in length. The limit of the diffraction pattern of such crystals is about 3 Å resolution.

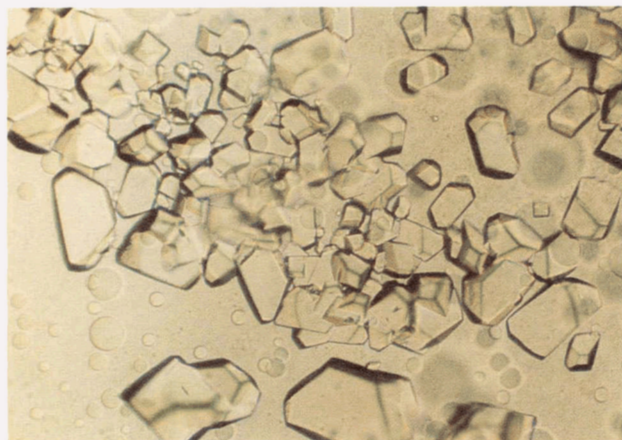


Fig. 3. Typical orthorhombic crystals of STMV grown in the laboratory on Earth. Note that many have quite irregular habits, aggregates are common, and the number of crystals per sample is generally high. Note as well that even though polarized light was used, the crystals show virtually no interference colors. Large examples of these crystals produce measurable diffraction intensities to about 2.3 Å resolution.

nomenon of protein crystal growth in microgravity is different than the same process in Earth's gravity.

Results

CRYOSTAT—STMV

The most dramatic results we achieved in any IML-1 experiments, with either CRYOSTAT or PCG, were from the samples of STMV that composed two chambers of the 20 °C, and a single sample of the temperature-ramped, CRYOSTAT sample containers (Table 1, A, B, and C, respectively). The crystals that are shown in Figure 4A,B were found to be quite remarkable. Upon initial examination of the chambers carrying the STMV samples, with the few minor but interesting exceptions discussed later, there were no crystals present except large and imposing specimens like those seen in Figure 4A,B. The mother liquor was otherwise clear. About six to eight of these were harvested from each chamber.

The STMV crystals recovered from the CRYOSTAT sample container maintained at constant 20 °C were quite uniform in habit, of almost perfect shape, but somewhat irregular at the two ends along the direction of the longest axis. We believe this irregularity to be principally due to premature termination of growth. This assertion is based on our previous time-lapse video microscopy studies of STMV crystal growth (Koszelak et al., 1991) and by extensive postmission microcrystalline growth that occurred in the crystallization chambers. The crystals recovered from the CRYOSTAT sample container that had experienced a temperature ramp were of similar size, marginally smaller, similar in number, but substantially more irregular at the two ends than those grown at 20 °C.

Table 1. CRYOSTAT crystallization solutions

Sample	Macromolecule	Precipitant	Buffer	Temperature (°C)
A. STMV	0.4 mL of 8 mg/mL virus in 20 mM Tris-HCl, pH 7.4	0.5 mL of 12.5% saturated (NH ₄) ₂ SO ₄ in H ₂ O	0.5 mL of 5.0% saturated (NH ₄) ₂ SO ₄ in H ₂ O	20
B. STMV	0.4 mL of 8 mg/mL virus in 20 mM Tris-HCl, pH 7.4	0.5 mL of 17.5% saturated (NH ₄) ₂ SO ₄ in H ₂ O	0.5 mL of 5.0% saturated (NH ₄) ₂ SO ₄ in H ₂ O	20
C. STMV	0.4 mL of 8 mg/mL virus in 20 mM Tris-HCl, pH 7.4	0.5 mL of 20.0% saturated (NH ₄) ₂ SO ₄ in H ₂ O	0.5 mL of 5.0% saturated (NH ₄) ₂ SO ₄ in H ₂ O	−4 to 17
D. Canavalin	0.4 mL of 25 mg/mL protein in H ₂ O plus trace NH ₄ OH	0.5 mL of 1.5× DPBS ^a , pH 6.8	0.5 mL of 0.5× DPBS, pH 6.8	20
E. Canavalin	0.4 mL of 25 mg/mL protein in H ₂ O plus trace NH ₄ OH	0.5 mL of 1.0× DPBS, pH 6.8	0.5 mL of 0.5× DPBS, pH 6.8	−4 to 17
F. Canavalin	0.4 mL of 25 mg/mL protein in H ₂ O plus trace NH ₄ OH	0.5 mL of 2.0× DPBS, pH 6.8	0.5 mL of 0.5× DPBS, pH 6.8	−4 to 17

^a DPBS is physiological saline: 0.7% NaCl, 50 mM Na₂PO₄/K₂PO₄, pH 6.8, plus 1 mM CaCl₂ and 1 mM MgCl₂. Multiplier, e.g., 1.5×, preceding DPBS implies that concentrations of all components of the DPBS are multiplied by this value.

The largest of the STMV crystals grown in CRYOSTAT was nearly 1.5 mm in length and 1.0 mm in both of the other two directions. This represents an increase in volume of considerably more than 10 times over that which we ever achieved in hundreds of crystal growth experiments on Earth. These were unquestionably the largest and most perfect STMV crystals we have ever grown. The STMV crystals (Table 1, A and B), were without visible defects. There were no striations, imperfections, aggregates, or twins. In addition, the quality and strength of the interference colors in polarized light (Wood, 1977) exceeded any we had previously observed.

Approximately 30 min to an hour after we began inspecting the STMV crystals from CRYOSTAT, the appearance and growth of small STMV crystals became evident, and shortly thereafter this light onset became pronounced. Thus, in Figure 4A,B, the heavy background of small crystals is in fact a consequence of post-mission growth and does not reflect what occurred in microgravity. Only the large crystals were products of that environment.

We noted that the large STMV crystals grown in all three of the CRYOSTAT chambers grew free of any contact with the interior surfaces of the sample containers. All were found freely suspended in the mother liquor, none was growing from any surface, and none of the crystals exhibited any anomaly in its shape that would suggest that it had grown in contact with either a chamber wall or another virus crystal.

CRYOSTAT—Anomalous forms

Of the few instances of irregular crystal growth one was a single aggregate of STMV crystals growing on the wall of the sample chamber that had experienced temperature ramping (Table 1, C). The crystals in this aggregate were

irregular in habit and imperfect in most other ways. This crystal aggregate was, we believe from its size, grown in microgravity. Another odd occurrence again involved the sample chamber that had been thermally ramped (Table 1, C). Very thin, highly irregular, shelflike crystals, not of very large size but clearly observable, were growing from one silicone rubber septum that sealed off the chamber from the outside. We could not further characterize these anomalous crystals except to say that they represent a flawed and unusual habit with which we are unfamiliar. We make note of these anomalies because it appears that the only imperfect and irregular STMV crystals grown in the CRYOSTAT were those that had direct contact with a surface.

Again, in the single CRYOSTAT chamber that was thermally ramped, we found six to eight STMV crystals of 0.2–0.4 mm that were of an entirely different morphology than the large cohabitant orthorhombic crystals. These crystals, seen in Figure 5, had an octahedral habit. Under polarized light they demonstrated no birefringence and extinguished completely when polarizer and analyzer were crossed. This behavior is characteristic of crystals belonging to the cubic class. Icosahedral viruses, such as STMV, frequently crystallize in cubic space groups since they embody this symmetry themselves. We therefore believe that crystals having such a novel unit cell were grown in this particular sample. We have not observed such crystals previously in the laboratory.

CRYOSTAT—STMV—X-ray

X-ray diffraction measurements have been recorded from perhaps 30 or more different STMV crystals grown in this laboratory. These data were collected for the purpose of solving the three-dimensional structure of the virus, and it includes heavy atom derivatized crystals and numerous

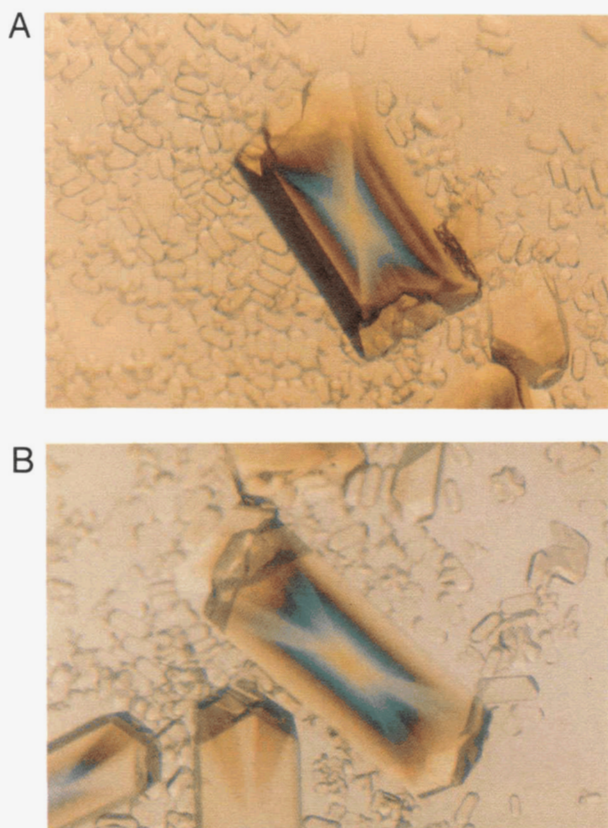


Fig. 4. Two examples of the unusually large (approximately $1.5\text{ mm} \times 1\text{ mm} \times 1\text{ mm}$) crystals of STMV grown in Cryostat by liquid-liquid diffusion at 20°C . Initially (4 h after recovery) only the large crystals were present in the chambers suspended in completely clear mother liquor. All of the smaller crystals formed during and after microscopic examination in the laboratory on Earth, demonstrating that crystallization was incomplete when the experiment was terminated. The irregularities on the ends of these crystals are another indication that growth had not gone to completion. The crystals are virtually flawless, unusual in itself for such large macromolecular crystals, the edges are sharp, no aggregates or anomalous habits were observed, and the striking interference colors under polarized light have never been observed in our laboratory on Earth. The diffraction patterns of these crystals extend to at least 1.8 \AA resolution, a substantial improvement over similar crystals grown on Earth.

low to moderate resolution data sets as well as high resolution data. We collected intensities from the best crystals available to us in order to optimize the means for structure determination. Thus, the best X-ray data that we had in hand at the time of the IML-1 mission would seem to represent the most rigorous control we could apply in assessing the relative quality of X-ray data collected from crystals grown in space. These data, then, were taken as the standard. The control for the space experiment was the “best” data we had ever collected from Earth-grown crystals.

X-ray diffraction data were recorded from eight different STMV crystals grown in the CRYOSTAT device aboard IML-1. These eight crystals were taken from the

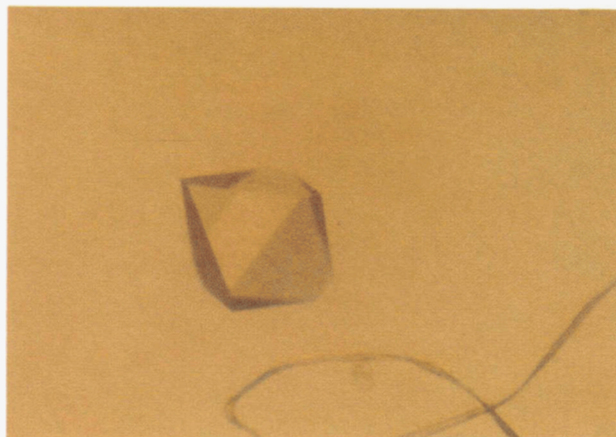


Fig. 5. One of several small but otherwise perfect STMV crystals having a unique octahedral habit that was grown, along with more familiar crystal forms, in the single CRYOSTAT STMV chamber that underwent thermal ramping. The maximum dimension of any of these crystals was about 0.3 mm . The crystals showed no evidence of birefringence under polarized light. No such crystals were observed in any other chambers in either PCG or CRYOSTAT nor, to our knowledge, have they been seen in a laboratory on Earth.

two chambers maintained at constant 20°C during the course of the flight (Table 1, A and B). The data were collected on an area detector system over a total period of 6 weeks and included nearly 95% of the possibly observable data between a resolution of 100 \AA and the high resolution limit of the diffraction pattern. The data set was composed of more than two and a half million intensities, which reduced upon symmetry merging to 295,000 independent structure amplitudes, or $F(hkl)s$. These data were compared with control data from six Earth-grown crystals whose data were previously recorded to the maximum resolution attainable.

A method of data comparison based on Wilson statistics (Wilson, 1949, 1970) has generally been used to compare the quality of different crystals or groups of crystals (DeLucas et al., 1989, 1991a,b; McPherson et al., 1991). The plot is essentially a measure of signal (structure amplitude) to noise (estimated error of the structure amplitudes) over the entire resolution range, or range of Bragg angles, for which the data were collected. The plot has two features that have been taken as a measure of comparative quality. The first is the general shape and distribution of the curve over the entire range, which serves as a measure of the signal-to-noise ratio for all resolutions. The second is the maximum resolution at which statistically significant data can still be observed. This latter value, somewhat arbitrary, is thought to serve as a measure of the inherent order of the packing of the molecules in the crystals.

In Figure 6 is seen the variation of the intensity-to-sigma ratio expressed as a function of the resolution for both the merged data from the six Earth-grown crystals

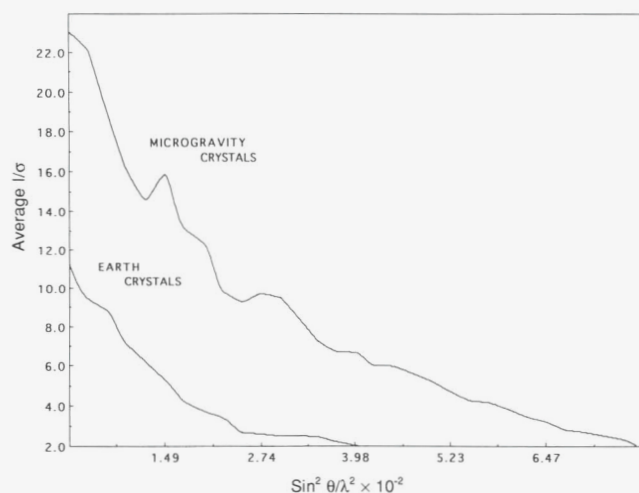


Fig. 6. The I/σ ratio for all STMV X-ray diffraction data for both Earth- and microgravity-grown crystals is plotted as a function of shells of equal size and increasing resolution, essentially $\sin^2 \theta / \lambda^2$, but stated in terms of the corresponding resolution. The estimated standard deviations were based on the deviations from the mean of symmetry-related and redundant measurements of individual reflections.

and that from the eight STMV crystals grown in microgravity. In addition, Table 2 presents the conventional merging residual R for the two data sets as a function of resolution. This provides a measure of the internal consistency of the data sets, and the number of reflections in each range greater than $I/2\sigma$.

Over the resolution range for which data were recorded the intensity-to-sigma ratio was consistently and substantially higher for the virus crystals grown in microgravity than for those grown in the laboratory. As previously observed for a number of protein crystals (DeLucas et al., 1989; McPherson et al., 1991), the differences are pronounced over the entire resolution range. The extent of the diffraction pattern, the maximum resolution for which useful data could be recorded, was significantly greater for crystals grown in microgravity as compared to those grown on Earth. This was not a marginal improve-

ment, but a clear and definite extension of the resolution. It represents an increase of nearly 50% in the amount of useful data available for high resolution analysis and refinement. Thus, the X-ray data collected from the STMV crystals grown in space represent a substantial improvement upon the quality and quantity of data otherwise available.

CRYOSTAT—Canavalin

In the two sample chambers (Table 1, E and F) of canavalin exposed to thermal ramping, almost identical results were obtained. In both cases, there were hundreds of small- to medium-sized crystals of canavalin, like those seen in Figure 7. These crystals were of the hexagonal prismatic habit that is commonly, but usually not exclusively, seen at colder temperatures in the laboratory.

The maximum size of the hexagonal crystals was no more than 0.3 mm and did not begin to approach in size those frequently grown in the laboratory. We suspect that the uniformly small size of these crystals was due both to the occurrence of uncontrolled nucleation in the samples and premature termination of crystal growth by completion of the space mission.

One unusual feature of these thermally ramped canavalin results is the absence of any rhombohedral crystals in the samples. Although the hexagonal prismatic form is favored by cold temperature, generally rhombohedral

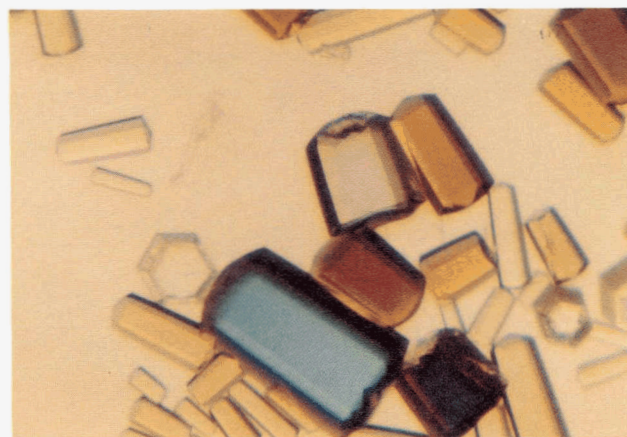


Fig. 7. Examples of hexagonal prismatic crystals of the protein canavalin grown on IML-1 in the thermally ramped sample container of the CRYOSTAT liquid-liquid diffusion device. The crystals are rather unexceptional in terms of both quality and size. Quality is good but not significantly better, and their sizes are considerably less than larger Earth-grown crystals. An interesting feature of these microgravity-grown crystals, however, that clearly distinguishes them from those grown on Earth is the shape of the void that forms along their unique axis. Whereas rapidly grown canavalin crystals on Earth (Fig. 2) frequently have a conical cusp extending inward from one end, these have a hexagonal lumen. This characteristic is presumably a consequence of pure diffusive transport in microgravity in contrast to the convective flow experienced by a crystal on Earth.

Table 2. Comparison of Earth and microgravity crystal X-ray data

Earth			Microgravity	
R_{merge}	No. reflections $>I/2\sigma$	Resolution (Å)	R_{merge}	No. reflections $>I/2\sigma$
10.24	55,553	3.2	7.10	48,444
19.84	47,467	2.5	7.81	48,010
25.89	24,541	2.1	11.59	48,581
25.85	9,431	1.9	16.06	38,020
27.36	3,765	1.8	20.97	24,406
12.64	140,757	Overall	8.92	207,461

crystals grow as well. The samples were exposed for at least several days to temperatures between 12 and 17 °C where the rhombohedral habit is strongly, almost exclusively favored, yet no rhombohedral crystals grew. This was not due to depletion of protein from the solution, since we were able to measure that remaining after sample recovery, and it was quite sufficient to permit additional crystal nucleation and growth. In fact, several days after sample recovery, rhombohedral crystals did begin growing in the two CRYOSTAT samples that had been temperature ramped but earlier contained only the hexagonal prismatic habit.

The hexagonal prismatic canavalin crystals grown in CRYOSTAT and seen in Figure 7 permitted an interesting observation. This pertained to the growth cusps that form along the unique axis of such crystals when they grow rapidly. In the laboratory, the cusps, seen clearly in Figure 2, form only at the growing end of the hexagonal canavalin crystals. These crystals grow asymmetrically in only one direction along the polar 6_3 axis in the crystals. The cusps are presumably a result of the failure of protein nutrient to reach the center as it is increasingly occluded by the more rapidly growing exterior faces.

The cusps in crystals grown on Earth are essentially conical in shape, as shown schematically in Figure 8. Many of the hexagonal prisms grown in space also show voids along the centers, but the cavities are not conical, they have the shape of hexagonal cylinders. Thus, the hexagonal prismatic canavalin crystals grown in microgravity, those that do demonstrate this effect, appear like empty hexagonal shell casings. We assume that the difference in cusp morphology is a demonstration that the recruitment of molecules into the growing crystal lattice in microgravity, where diffusion is the sole means of

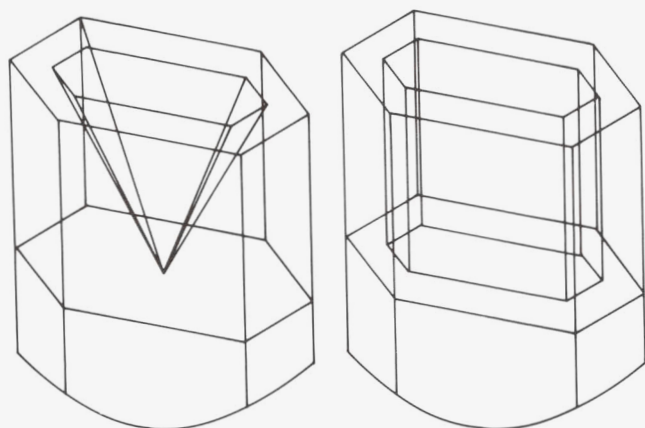


Fig. 8. On the left is a diagram showing the shape of the empty cusp that forms along the central prismatic axes of hexagonal canavalin grown rapidly at 4 °C on Earth. On the right is a similar crystal grown in microgravity showing the hexagonal cylindrical vacuole that is uniformly seen in space-grown crystals. The difference in this cusp shape presumably reflects the pure diffusive transport that exists in space in contrast to the convective mixing that occurs in Earth's gravity.

transport, is significantly different than in the laboratory where convective mixing predominates.

PCG—STMV

STMV crystals grown at 22 °C in the PCG instrument (Table 3, entries o–r) are shown in Figure 9. They have the expected habit for this temperature range, that of orthorhombic prisms. The crystals have a more or less continuous size distribution extending from a few microns to about 0.6 mm in their longest dimension. Many of these are comparable to the largest that we have previously grown in the laboratory but do not significantly exceed that size. As with canavalin, the major difference between these STMV crystals and those we routinely produce in the laboratory (see Fig. 3) is the uniform quality. The STMV crystals grown in the PCG device are virtually defect free and of consistent morphological perfection. There are no aggregates, as is common on Earth, and the edges and faces are near exact throughout.

Of the PCG experiments, the results for STMV at 4 °C were the most unanticipated. Rather than the orthorhombic prisms seen in Figure 9 at 22 °C, and certainly most generally seen at 4 °C on Earth as well, a large number of very large, thin, hexagonal plate crystals, like those shown in Figure 10, were observed. Although smaller examples of these are occasionally seen at low temperature,



Fig. 9. Crystals of STMV grown by vapor diffusion in a sample chamber of the PCG device aboard IML-1. The crystals are entirely representative of those seen in all of the chambers containing STMV at 22 °C. Like the canavalin crystals shown in Figures 10 and 11, these crystals were grown in 40- μ L suspended drops over an 8-day period. The crystals are virtually flawless, of uniformly perfect habit. Aggregates, twins, and anomalous habits are nearly absent, and all faces are well developed. Although the habits are the same as seen on Earth, the crystals demonstrate more pronounced interference colors under polarized light. The largest crystals seen here are on the order of 0.6 mm in their longest direction. The size distribution, as with canavalin, is essentially continuous from very small to the largest crystal observed. This continuum of sizes is distinctly different than was observed for STMV crystals grown by liquid–liquid diffusion methods in CRYOSTAT.

Table 3. PCG crystallization solutions

Sample	Macromolecule	Cell	Precipitant (1 mL)	Buffer ^a (μL)	Temperature (°C)
Cells g–k: canavalin	20 μL of 25 mg/mL protein in H ₂ O plus trace NH ₄ OH	a	0.5× DPBS, pH 6.8	20	4
		b	1.0× DPBS, pH 6.8	20	4
		c	1.5× DPBS, pH 6.8	20	4
		d	2.0× DPBS, pH 6.8	20	4
		e	1.0× DPBS, pH 6.8	20	4
Cells l–o: canavalin	20 μL of 25 mg/mL protein in H ₂ O plus trace NH ₄ OH	f	Same as a	20	20
		g	Same as b	20	20
		h	Same as c	20	20
		i	Same as d	20	20
Cells p–t: STMV	20 μL of 8 mg/mL virus in 20 mM Tris-HCl, pH 7.4	j	10.0% saturated (NH ₄) ₂ SO ₄ in H ₂ O	20	4
		k	12.5% saturated (NH ₄) ₂ SO ₄ in H ₂ O	20	4
		l	15.0% saturated (NH ₄) ₂ SO ₄ in H ₂ O	20	4
		m	17.5% saturated (NH ₄) ₂ SO ₄ in H ₂ O	20	4
		n	20.0% saturated (NH ₄) ₂ SO ₄ in H ₂ O	20	4
Cells u–x: STMV	20 μL of 8 mg/mL virus in 20 mM Tris-HCl, pH 7.4	o	Same as j	20	20
		p	Same as k	20	20
		q	Same as l	20	20
		r	Same as m	20	20

^a Buffer syringe barrel for each sample was the same as the precipitant solution. Thus, all vapor diffusion experiments were initially at one-half the precipitant concentration upon activation.

and usually accompanied by the prismatic habit, we have never seen them to appear in such number or size, to the exclusion of the orthorhombic prisms and after such a limited growth period.



Fig. 10. Typical examples of the crystals of STMV obtained in all of the sample chambers of the PCG device maintained at 4 °C. The habit is that of very large, thin, hexagonal plates that show no interference colors. These crystals are of exceptional quality as judged by visual inspection, almost flawless. Again there are no aggregates, secondary growth, or twins. The largest crystal seen in this photograph is about 1.5 mm across its face, a smaller crystal lies on top of it. This is an uncommon habit, is seen only very infrequently in the laboratory, and is nearly always accompanied by crystals of the more common orthorhombic form. No crystals of this morphology were seen in any of the IML-1 PCG samples at 22 °C.

The hexagonal plates, which in some cases have thicknesses of about 0.15 mm, often have face diameters that exceed 1.5 mm. These are extremely large by Earth standards and surpass any that we have seen in the laboratory. The overall quality and absence of defects in these crystals is again impressive. The apparent lines and striations that one sees in Figure 10 are, in fact, not flaws in the crystals, but are produced by sedimentation, in gravity, of many other smaller thin plates atop the largest. The absence of aggregates and secondary growth from the crystals suggests that each grew unperturbed in its local environment and was little influenced by neighbors.

A preliminary X-ray diffraction analysis was carried out on the hexagonal plate crystals to establish their symmetry properties. This showed that the crystallographic unit cell was not that commonly found, but a derivative having monoclinic symmetry I2 (equivalent of space group C2) with dimensions $a = 175.73 \text{ Å}$, $b = 170.00 \text{ Å}$, $c = 244.48 \text{ Å}$, and $\beta = 92.66^\circ$. This cell is similar to the body-centered orthorhombic crystal except that the coincidence of two viral dyad axes with twofold crystallographic axes is lost. The solvent content of the monoclinic unit cell is about 5% greater than that of the orthorhombic unit cell.

PCG—Canavalin

The 22 °C refrigerator/incubator module (R/IM) of PCG yielded crystals of canavalin of reasonable size and excellent visual quality. PCG-grown canavalin crystals (Ta-

ble 3, entries f-i) having the rhombohedral unit cell expected for this temperature range are shown in Figure 11. Apparent in these samples is the uniform clarity of the crystals; their morphological quality; the general absence of defects, striations, twins, or aggregates; the sharp edges and precise angles; the lack of anomalous shapes; the relatively strong birefringence; and their uniformity of size. Although none of the crystals exceed in size those commonly grown in the laboratory, the consistency of this high degree of quality is exceptional for canavalin crystals.

The X-ray diffraction results for canavalin crystals were not as impressive as for STMV. The largest crystals of canavalin grown on the IML-1 mission were those of rhombohedral habit produced in PCG at 22 °C (Table 3, f-i). Although of good size, and quite adequate for data X-ray analysis, they were not comparable in size to those we commonly use for data collection in the laboratory. Nonetheless, data were collected from one PCG-grown crystal, chosen from among the largest that had grown. The results of the canavalin analysis were for all practical purposes the same as those reported for canavalin in previous space experiments employing the PCG instrument (DeLucas et al., 1991a,b; McPherson et al., 1991). As with other microgravity-grown canavalin crystals, the X-ray data demonstrated an improvement of signal to noise over the entire resolution range when compared with the "best" Earth-grown crystal data. There was, however, no significant extension of the diffraction pattern.

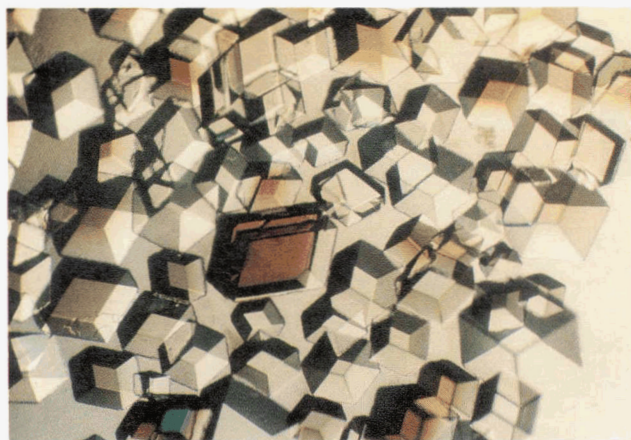


Fig. 11. Rhombohedral crystals of the protein canavalin grown in a sample chamber of the PCG device during IML-1. These are quite typical of the results in all chambers containing canavalin at 22 °C. The quality of the crystals is uniformly exceptional when compared with crystals grown on Earth. Defects are rare or entirely absent, edges sharp, faces flawless, interference colors strong, and aggregates or twins virtually absent, as are anomalous habits. The results seen here are practically the same as those seen with canavalin on previous Space Shuttle experiments.

Failures

Some samples were outright failures in that no crystals were grown or that crystals of such small size or poor quality appeared that any further consideration was fruitless. The single canavalin sample in the CRYOSTAT container (Table 1, entry D) that was maintained at 20 °C produced no crystals that were visible 4 h after Shuttle landing. Control samples run in parallel on the ground produced good quality rhombohedral crystals of reasonable size. In the same category were all the canavalin samples at 4 °C in the PCG experiment (Table 3, entries a-e). In each of these samples thousands of microcrystals appeared. The crystals exhibited the expected cold temperature habit of hexagonal prisms, but none was more than a few microns in size. Rehearsal experiments did not suggest this result nor did it reflect the ground controls.

Conclusions

The IML-1 experiments indicate that macromolecular crystallization in microgravity exhibits some unique and novel characteristics. We observed a reduction in visible defects for nearly all samples, a substantial increase in size for STMV crystals grown in CRYOSTAT, and altered habits were seen for STMV crystals grown in PCG at 4 °C. We noted a novel octahedral crystal form of STMV in CRYOSTAT at low temperature, a change in cusp form in canavalin crystals in CRYOSTAT, and a uniform absence of secondary nucleation and crystal aggregation. The results suggest that the kinetics of crystallization are different, which in turn alters growth rates and the final size of crystals. In general, the optical properties of crystals were enhanced and the diffracting power over the entire resolution range for both canavalin and STMV was improved. There was a striking increase in the resolution limit for STMV crystals grown in CRYOSTAT. Most of the observations suggest that crystallization is not only different in space, but that it favors improved macromolecular crystal growth, not the contrary.

Some peripheral factors that only slightly influence the outcome of crystallization on Earth appear to gain increased importance in microgravity. An example is the influence of surfaces on nucleation and growth. For canavalin grown in PCG at 20 °C, which yielded the best results for that particular protein, the largest canavalin crystals of all were those that nucleated and grew on small fibers of rare occurrence that contaminated the samples. This is seen in Figure 12. Although heterogeneous nucleation of canavalin on such fibers has been seen on Earth, it does not usually have any bearing on final size. Similarly, the only crystal aggregates observed in the samples were seen to be associated with nucleation on the chamber walls or the rubber septa. Temperature, which has some influence on the growth of both canavalin and STMV on Earth seemed also to have had its ef-

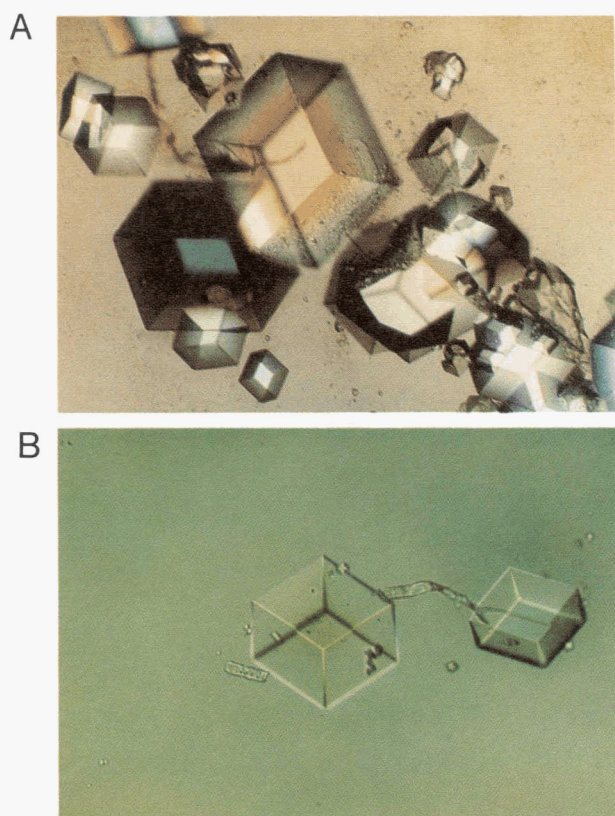


Fig. 12. Two examples of rhombohedral canavalin crystals grown in the PCG experiment at 20 °C that have nucleated and grown on small fibers present in the crystallization samples. The largest crystal in **a** is the largest crystal observed for any canavalin sample and is about 0.5 mm on an edge. In both **a** and **b** the small fibers are actually embedded in the crystals, and in **b** the two crystals, though otherwise perfect, are joined by a fiber link.

fect amplified in space. The resulting differences, in both PCG and CRYOSTAT for canavalin and STMV, at low and warm temperature in terms of habit, size, and other features were more striking in nearly all cases than for control samples in the laboratory.

The influence of instrument and method was pronounced. Different results were obtained from PCG and CRYOSTAT from identical samples of protein and virus. As on Earth, crystallization in space proceeds in a variety of ways depending on the means employed to bring the system to equilibrium. It seems unlikely, from the results of these experiments, that a single method will prove uniquely superior. On this one mission, for example, the best canavalin crystals were grown by vapor diffusion in PCG, whereas the best STMV crystals came from the liquid-liquid diffusion technique embodied in CRYOSTAT.

In terms of the kinetics, or time dependence of the crystallization process in space, there appears to be a more complicated relationship than we might have expected. It seems to impact not only the growth rate of crystals once they have nucleated, but the nature of the nucleation events themselves. For example, it appears

that in vapor diffusion the equilibration between a protein microdroplet and its reservoir occurs more rapidly in microgravity than on Earth. This is reflected by the extent of nuclei formation and the altered optima for precipitant concentration, pH, or temperature. This was seen most dramatically in the microcrystalline showers of canavalin crystals in the PCG experiments at 4 °C as well as the anomalous habits of the PCG-grown STMV crystals at that same temperature. The results suggest that in the PCG samples, at both temperatures, equilibration and crystal growth were essentially complete by the end of the mission.

For liquid-liquid diffusion in the CRYOSTAT, quite the contrary appears to be true. Nucleation was less pronounced, particularly in the case of STMV, and growth was significantly slower than on Earth. From the irregular edges at the ends of the otherwise perfect STMV crystals grown in CRYOSTAT, a characteristic of incomplete growth, to the appearance of vast showers of microcrystals hours after the samples were retrieved, we can be certain that the CRYOSTAT experiment suffered from premature termination. Perhaps even larger and more regular crystals might have been grown had the experiment continued in microgravity for a longer period of time. Premature termination of growth was more severe in the case of CRYOSTAT STMV crystals grown under thermally ramped conditions than those maintained at 20 °C. This was seen by the even more irregular appearance of the edges of those crystals. This is consistent with the later nucleation and slower growth of the virus crystals due to the reduced diffusivity of the ammonium sulfate ions at the lower temperature and the retrograde solubility of the virus in high salt concentrations as a function of temperature.

Analyses of the X-ray diffraction measurements, particularly those for STMV crystals grown in CRYOSTAT, yielded evidence consistent with qualitative observations that protein crystals can be grown in a microgravity environment that have improved diffraction properties. This argument has been advanced previously (Littke & John, 1986, 1988; DeLucas et al., 1989, 1991a,b; McPherson et al., 1991) and the results we obtained here are in agreement with those reports. Finally, we note that the X-ray diffraction data collected from the STMV crystals grown in space in the CRYOSTAT will be put to practical use. These data will permit the refinement of the structure of the STMV, now in progress, to a resolution not before possible, to a resolution higher than has ever before been achieved for any crystalline virus.

Materials and methods

In Earth's gravity

Canavalin, a protein of about $M_r = 145,000$, consisting of three identical subunits related by a triad axis of sym-

metry, is the major storage protein of the jack bean, *Canavalia ensiformis*. It is typical of all legume storage proteins composing what is traditionally called the vicilin class. The protein, whose characteristics are described elsewhere (Sumner & Howell, 1936; Smith et al., 1982; Gibbs et al., 1989; Ng et al., 1992a,b) was purified from jack bean meal by conventional procedures and was recrystallized four times before use in the microgravity experiments.

Canavalin can be grown in at least four different crystal forms under various conditions of salt concentration, pH, and temperature (McPherson & Spencer, 1975; Ko et al., 1992). The predominant form is rhombohedral, of space group R3, with equivalent hexagonal unit cell dimensions of $a = b = 136.8 \text{ \AA}$ and $c = 75.7 \text{ \AA}$. At temperatures in the neighborhood of 0–10 °C, it frequently crystallizes in the hexagonal space group P6₃ with $a = b = 126.35 \text{ \AA}$ and $c = 51.64 \text{ \AA}$. The habits are rhombic and hexagonal prisms, respectively. The protein can be crystallized in from 6 h to several days depending on conditions. On Earth, canavalin crystals have commonly been grown that are up to a millimeter on a side, in the case of the rhombohedral crystals, and over 2 mm long for the hexagonal prisms like those seen in Figure 2.

Canavalin can be readily crystallized in the rhombohedral form by bringing a 25–30-mg/mL protein solution to pH 6.8 in the presence of 0.5–2.0% NaCl at 12–37 °C. At 2.0–4.0% NaCl and 0–10 °C the hexagonal or orthorhombic form commonly grows. Just as often, however, under these same conditions the rhombohedral form occurs as well. Often, transformations between the forms are observed with the rhombohedral habit generally being that which persists. The cubic form of canavalin very seldom is seen, and it appears to be specific to individual preparations of the protein.

Canavalin crystals are frequently flawed, often twinned, and in spite of their size, do not yield diffraction data to very much beyond 3 Å resolution. Some data are generally present beyond this limit, but only a relatively small percentage of that possibly observed. This appears to be true of all of the crystal forms of canavalin. Some improvement in the quality of the rhombohedral canavalin crystal diffraction pattern, as measured by relative Wilson statistical analyses (Wilson, 1949, 1970), has been achieved in previous microgravity experiments, but no significant extension of its diffraction pattern to higher resolution has so far been observed (DeLucas et al., 1991a,b; McPherson et al., 1991).

Satellite tobacco mosaic virus is the T = 1 icosahedral satellite virus to the classic rod-shaped tobacco mosaic virus, TMV. It was discovered in 1985 by Professor Alan Dodds at the University of California at Riverside and was characterized by him both physically and genetically (Dodds, 1991). It has a molecular weight of about two million Daltons, a capsid composed of 60 identical protein units of mass $M_r = 17,500$, a genome of 1,059 bases

of single-stranded RNA that code for the coat protein alone, plus, possibly, one other small protein. It requires coinfection by TMV in order to replicate.

The virus was purified from leaves of tobacco plants infected at 3 weeks with TMV plus STMV and harvested after a growth period of an additional 3–6 weeks. Gradient density centrifugation provides the principal method for its isolation (Valverde & Dodds, 1986, 1987), whereas recrystallization from ammonium sulfate solutions allows the preparation of highly purified samples. The virus can be obtained in hundred-milligram amounts and reproducibly crystallized under a variety of conditions. Its entire three-dimensional structure has recently been determined by X-ray diffraction analysis in this laboratory (Larson et al., 1992).

STMV crystals can assume a variety of habits, though by far the most common is that of orthorhombic prisms like those seen in Figure 3. All STMV crystals that we have grown on Earth have, regardless of habit, shown themselves by X-ray diffraction analysis to be of space group I222 with $a = 174.23 \text{ \AA}$, $b = 191.74 \text{ \AA}$, and $c = 202.51 \text{ \AA}$. As with canavalin, extensive X-ray diffraction data had been collected on many STMV crystals in the course of its structure determination. Because of its manageable unit cell size, X-ray data from the virus crystals, as for canavalin, could be collected without difficulty on an area detector system. Data existed prior to the execution of the IML-1 mission to the maximum extent of the respective diffraction patterns. This was, for most practical purposes, about 2.8 Å resolution for canavalin and 2.3 Å for the STMV crystals.

In the laboratory, STMV crystals are commonly grown (Koszelak et al., 1989) by exposing a 5–8-mg/mL virus solution to concentrations of ammonium sulfate ranging from 10 to 20% saturation at pH between 6.0 and 7.5. The crystals can be grown in a few days at room temperature and have been the subject of extensive investigation of the macromolecular crystallization process using inelastic light-scattering techniques (Malkin et al., 1993; Malkin & McPherson, 1993). At 4 °C, under otherwise identical conditions, the habit of Figure 3 is also generally observed but flat hexagonal plates are occasionally seen as well. Normally, this latter habit requires several weeks or even months to grow.

X-ray diffraction data for both laboratory- and space-grown crystals were collected in identical fashions so far as we were able. The area detector system used was a San Diego Multiwire Systems (SDMS) (Xuong-Hamlin) two panel arrangement with a two theta table. The X-ray source was a Rigaku RU-200 rotating anode generator fitted with a Supper graphite crystal monochromator to produce CuK $_{\alpha}$ radiation. The goniostat was a Huber three circle instrument. The X-ray diffraction data were, in general, collected from a single crystal for no more than 4–5 days. This eliminated most decay problems for these otherwise very stable crystal specimens. The X-ray

diffraction data were processed using the programs of C. Nielson as provided to us by SDMS and run on a Silicon Graphics computer. All statistical measures reported in this paper are derived directly from that program system.

For the canavalin X-ray data collection, helium-filled boxes were used having a pathlength of 615 mm from crystal to detector face. For the STMV crystals, boxes of pathlength 944 mm were inserted. Data were generally collected by rotating the crystal from -30° to $+30^\circ$ in omega at appropriate chi and phi settings. The frame size varied from 0.10° to 0.15° but was usually 0.12° with counting times ranging from 2 to 4 min.

In microgravity

The CRYOSTAT device, built by Kayser-Threde GMBH of Munich, Germany, consists of two isothermal enclosures, each coupled to a motorized drive used to activate the experiment on command. Within each enclosure was placed a lucite sample container, shown schematically in Figure 13, and prepared for flight in Figure 14. Each container can accommodate seven protein samples, three on one side of the lucite block and four on the other, so that the entire CRYOSTAT experiment within the two enclosures carried a total of 14 samples. Each experimental sample consisted of two opposing 0.5-mL chambers that were filled with protein and precipitant solutions through ports that were then sealed by rubber septa. The two chambers of each sample were segregated by a lucite partition capable of sliding upon experiment activation.

The lucite partition had, for each pair of chambers, a hole displaced to the side, and the volume of this gap in the partition was filled with a buffer solution provided by a third buffer chamber. Thus, when the slide was moved approximately 1 cm, the buffer-filled hole in the slide became positioned between the protein and precipitant chambers so that a three-phase system was created. The three phases were, sequentially, a 0.5-mL chamber of protein solution interfaced to a 2.0-mm-thick layer of buffer, which in turn interfaced directly to the 0.5-mL chamber of the precipitant solution. Activation of the slide mechanism occurred simultaneously for all sample experiments approximately 8 h after launch of the Space Shuttle Columbia at 9:45 a.m. (EST) on January 22, 1992. The two sample containers were not treated identically during the CRYOSTAT experiment. In one isothermal chamber the temperature was maintained constant throughout at 20°C for the 8 days of the mission. The second container, however, was held initially at -4°C . Upon activation the temperature was raised to 0°C over a 2-h time period. During the remaining 7 days of the mission, the temperature was increased in a linear manner with time from 0 to 17°C . The temperature profile of the thermally ramped sample container over the course of the mission is shown in Figure 15.

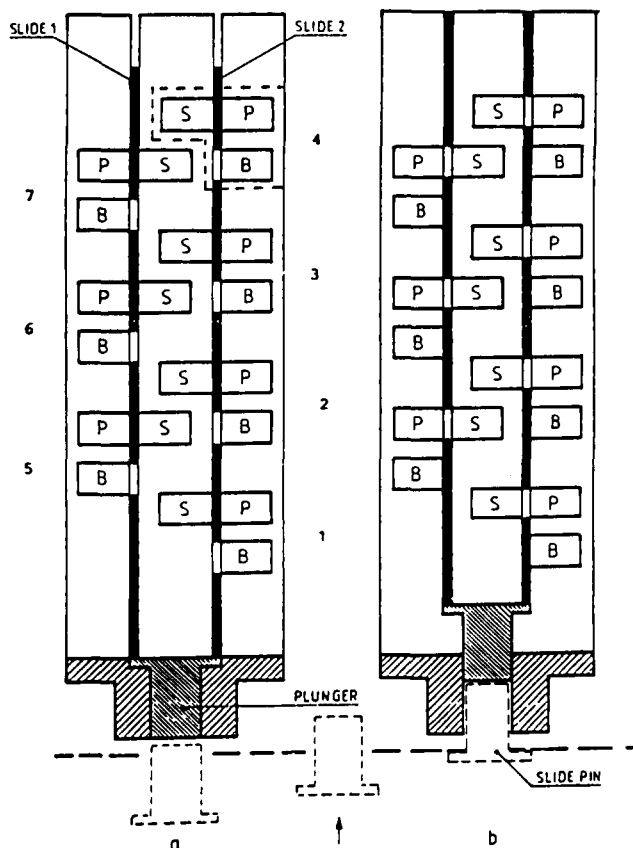


Fig. 13. A schematic diagram of the sample container for the CRYOSTAT instrument showing the three crystallization reactors on the left and four on the right, each composed of a protein, precipitant, and buffer chamber. The activation plunger on the left moves to drive the partitioning slides toward the rear, as on the right, to establish an interface between the protein and precipitant fluids.

During execution of the experiment, the sample chambers were placed in a Shuttle middeck locker approximately 12 h before launch in a carrier container that maintained their temperature at -4°C until a space environment was achieved. About 8 h following launch the sample containers were removed from their container in the middeck locker and inserted into their respective isothermal enclosures within the CRYOSTAT instrument itself. Shortly thereafter the experiment was initiated. Until the end of the mission the instrument was essentially passive except for its temperature control and logging functions. Approximately 18 h prior to reentry, astronaut Bondar opened the isothermal enclosures, removed the lucite sample containers, and transported them back to the middeck where they were returned to their constant temperature carrying container, then at ambient temperature. The total elapsed time for undisturbed crystallization to occur was about 164 h. The Shuttle landed at Dryden Space Facility at 8:12 a.m., January 29, 1992.

The sample containers were transported and handed over to the investigators approximately 2 h after landing.

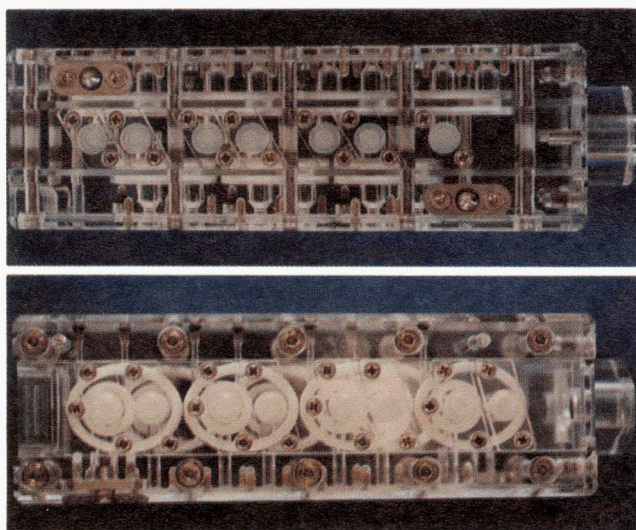


Fig. 14. Two views of one of the two lucite protein sample containers utilized by CRYOSTAT. Photograph above is a view of the top with rubber septa sealing seven precipitant chambers. The protein and buffer chambers can also be seen along the two sides of the container in pairs, three pairs on one side and four pairs on the other. The photograph below shows the side of the sample container having four pairs of chambers and the system of teflon seals that interface the lucite block to the sliding partition that separates the protein from the precipitant prior to activation.

The sample containers in their isothermal carrier were then transported to the investigator's laboratory at the University of California at Riverside, the crystals removed from the CRYOSTAT sample containers, subjected to microscopic analysis, and photographed less than 5 h after the Shuttle had landed. Crystals were mounted in quartz capillaries by conventional means for X-ray diffraction analysis during that same period.

The CRYOSTAT experiment included three samples of canavalin and three samples of STMV and the solution parameters for all chambers, one of which is shown in

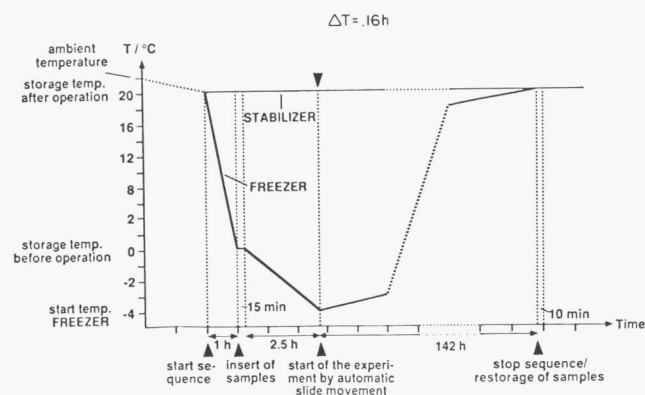


Fig. 15. A diagram of the temperature profile during the IML-1 mission of the CRYOSTAT sample container undergoing thermal ramping. The second sample container was maintained at a constant 20 °C.

Figure 16, are shown in Table 1. In addition to the experiments described here, four chambers in the constant temperature container were used to carry out an investigation of the crystallization in space of the bacterial rhodopsin protein under the direction of the second of three principal investigators, Professor Gottfried Wagner of The University of Giessen, Giessen, Germany. Four chambers were similarly used in the temperature ramped container with the protein beta-galactosidase. This study was under the direction of Professor Walter Littke, Albert-Ludwigs Universität, Freiburg, Germany. The results of their experiments will be presented in separate reports. The temperature ramping of one sample container during the course of the mission was to promote crystallization of the beta-galactosidase and was not instituted to produce any specific effects in the cases of canavalin or STMV. As is evident here, space experiments are nearly always multi-investigator and must accommodate a range of samples and experimental conditions. Thus, some compromise of this nature is common.

No attempt will be made here to describe the instrument or elaborate on the procedures employed in the PCG experiment (instrument built by Teledyne-Brown Engineering Co., Huntsville, Alabama), these have been described elsewhere before (DeLucas et al., 1986, 1989, 1991a,b). Some features of the PCG experiment on this particular mission deserve attention. On IML-1, two PCG thermal enclosures were flown, one maintained at a constant 22 °C and the second maintained at 4 °C. Each thermal enclosure (designated an R/IM [refrigerator/incubator module]) carried a total of 60 samples, or 120 total for the mission. There were 15 different proteins

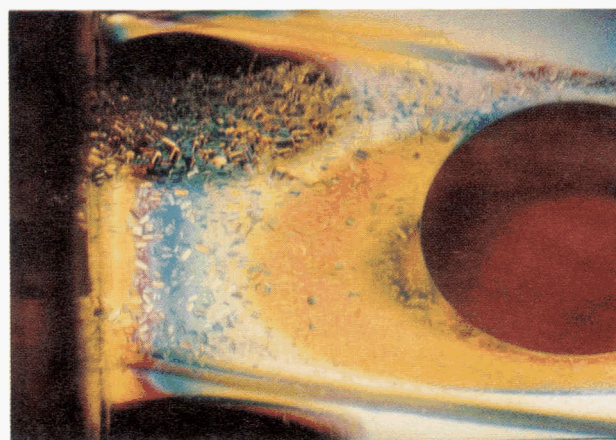


Fig. 16. Photograph made under a low power dissecting microscope of one of the crystallization chambers in the lucite sample container (total volume protein plus precipitant = 1 mL) of the CRYOSTAT device. The mass of small protein crystals seen here are the canavalin crystals grown on IML-1 in the thermally ramped container. The large round object at the right is the aperture of the filling port for the precipitant chamber.

making up the PCG complement. Canavalin and STMV each occupied five chambers in the 4 °C R/IM and four chambers each in the 20 °C R/IM for a total of 18 experiments. As indicated in Table 2, microdrop size for both canavalin and STMV was 40 μ L and consisted of equal parts of the protein and precipitant solutions. The reservoirs were 1 mL in volume. Identical samples were flown in parallel in both the 4 and 22 °C R/IMs.

The PCG experiment was activated and deactivated on about the same schedule as the CRYOSTAT experiment, although it was carried in the middeck of the Shuttle rather than in the Spacelab module. Although the PCG sample containers were returned to investigators at nearly the identical time, for logistical reasons, a consequence of the many coinvestigators and the necessity for a central distribution point (which was Birmingham, Alabama), we did not have laboratory access to our PCG protein crystal samples for about 60 h following landing of the Shuttle.

It is important to emphasize that the canavalin and STMV samples used in the CRYOSTAT and the PCG experiments were identical. They were both from the same preparations that were carried to Cape Kennedy loading and launch facilities and there divided for the two experiments. The samples, prior to loading in the CRYOSTAT and PCG sample containers were centrifuged and filtered through 0.22 μ m membranes and maintained at 4 °C throughout all subsequent manipulations in laboratories at Cape Kennedy.

Upon return to the laboratory at the University of California at Riverside (UCR), crystals grown in both the CRYOSTAT and PCG experiments aboard IML-1 were examined under polarized light using Olympus microscope systems (models SZH and BH). Photographs were made on Ektachrome 100 film with the former system and using an Olympus OM-2 35-mm camera on the latter, all photographs being taken in automatic mode. As noted above, the CRYOSTAT crystals were recorded within 6 h after Shuttle landing, those from PCG about 60 h later. Control experiments using, again, the same identical samples of protein and virus were carried out at UCR using alternate CRYOSTAT sample containers along with a temperature programmable refrigerator. Laboratory mockups of the PCG instruments permitted us to duplicate those microgravity experiments in the laboratory as well. These control experiments were carried out during the course of the mission, though they were initiated and terminated about 24 h after the equivalent events in space.

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References

- DeLucas, L.J. & Bugg, C.E. (1987). New directions in protein crystal growth. *Trends Biotechnol.* 5, 188–192.
- DeLucas, L.J., Smith, C.D., Carter, D.C., Snyder, R.S., McPherson, A., Koszelak, S., & Bugg, C.E. (1991a). Microgravity protein crystal growth; results and hardware development. *J. Crystal Growth* 109, 12–16.
- DeLucas, L.J., Smith, C.D., Smith, H.W., Senadhi, V.K., Senadhi, S.E., Ealick, S.E., Bugg, C.E., Carter, D.C., Snyder, R.S., Weber, P.C., Salemme, F.R., Ohlendorf, D.H., Einspahr, H.J., Clancy, L., Navia, M.A., McKeever, B.M., Nagabhushan, T.L., Nelson, G., Babu, Y.S., McPherson, A., Koszelak, S., Stammers, D., Powell, K., & Darby, G. (1989). Protein crystal growth in microgravity. *Science* 246, 651–654.
- DeLucas, L.J., Smith, C.D., Smith, W., Senadhi, V.K., Senadhi, S.E., Ealick, S.E., Carter, D.C., Snyder, R.S., Weber, P.C., Salemme, F.R., Ohlendorf, D.H., Einspahr, H.M., Clancey, L.L., Navia, M.A., McKeever, B.M., Nagabhushan, T.L., Nelson, G., McPherson, A., Koszelak, S., Taylor, G., Stammers, D., Powell, K., Darby, G., & Bugg, C.E. (1991b). Protein crystal growth results for shuttle flights STS-26 and STS-29. *J. Crystal Growth* 110, 302–311.
- DeLucas, L.J., Suddath, F.L., Snyder, R., Naumann, R., Broom, M.B., Pusey, M., Yost, V., Herren, B., Carter, D., Nelson, B., Meehan, E.J., McPherson, A., & Bugg, C.E. (1986). Preliminary investigations of protein crystal growth using the Space Shuttle. *J. Cryst. Growth* 76, 681–693.
- Dodds, J.A. (1991). Structure and function of the genome of satellite tobacco mosaic virus. *Can. J. Plant Pathol.* 13, 192–195.
- Erdmann, V.A., Lippmann, C., Betzel, C., Dauter, Z., Wilson, K., Hilgenfeld, R., Hoven, J., Liesum, A., Saenger, W., Muller-Fahrnow, A., Hinrichs, W., Duvel, M., Schulz, G., Muller, C.W., Wittmann, H.G., Yonath, A., Weber, G., Stegen K., & Plaas-Link, A. (1989). Crystallization of proteins under microgravity. *FEBS Lett.* 259, 194–198.
- Gibbs, P.E.M., Strongin, K.B., & McPherson, A. (1989). Evolution of legume seed storage proteins—A domain common to legumins and vicilins is duplicated in vicilins. *Mol. Biol. Evol.* 6, 614–623.
- Giege, R., Lorber, B., Mikol, V., Moras, D., Ruff, M., Theobald A., & Thierry, J.C. (1988). *Bull. Inst. Pasteur* 86, 9–20.
- Ko, T.-P., Ng, J.D., Day, J., Greenwood, A., & McPherson, A. (1992). The three dimensional structure of jack bean canavalin. *Plant Physiol.*, in press.
- Koszelak, S., Dodds, J.A., & McPherson, A. (1989). Preliminary analysis of crystals of satellite tobacco mosaic virus (STMV). *J. Mol. Biol.* 208, 323–326.
- Koszelak, S., Martin, D., Ng, J., & McPherson, A. (1991). Protein crystal growth rates determined by time lapse microphotography. *J. Crystal Growth* 110, 177–181.
- Koszelak, S. & McPherson, A. (1988). Time lapse microphotography of protein crystal growth using a color VCR. *J. Crystal Growth* 90, 340–343.

- Larson, S.B., Koszelak, S., Day, J., Greenwood, A., & McPherson, A. (1992). Double helical RNA in satellite tobacco mosaic virus. *Nature*, in press.
- Littke, W. (1985). Abstracts of the 17th Aerospace Sciences Meeting, New Orleans, paper 79-0311.
- Littke, W. (1988). Protein single crystal growth under microgravity. *J. Crystal Growth* 90, 344-348.
- Littke, W. & John, C. (1984). Protein single crystal growth under microgravity. *Science* 225, 203.
- Littke, W. & John, C. (1986). Protein single crystal growth under microgravity. *J. Crystal Growth* 76, 663-672.
- Malkin, A.J., Cheung, J., & McPherson, A. (1993). Crystallization of satellite tobacco mosaic virus I. Nucleation phenomena. *J. Crystal Growth*, in press.
- Malkin, A.J. & McPherson, A. (1993). Crystallization of satellite tobacco mosaic virus. II. Postnucleation events. *J. Crystal Growth*, in press.
- McPherson, A. (1976). The growth and preliminary investigation of protein and nucleic acid crystals by X-ray techniques. In *Methods of Biochemical Analysis*, Vol. 23 (Glick, D., Ed.), pp. 249-345. Academic Press, New York.
- McPherson, A. (1982). *Preparation and Analysis of Protein Crystals*. John Wiley, New York.
- McPherson, A. (1990). Current approaches to macromolecular crystallization. *Eur. J. Biochem.* 189, 1-23.
- McPherson, A. (1991). Useful principles for the crystallization of proteins. In *CRC Reviews of Membrane Protein Crystallization* (Hartmut, M., Ed.), pp. 1-53. CRC Press, Boca Raton, Florida.
- McPherson, A., Greenwood, A., & Day, J. (1991). The effect of microgravity on protein crystal growth. *Adv. Space Res.* 11, 343-356.
- McPherson, A. & Rich, A. (1973). X-ray crystallographic study of the quaternary structure of canavalin. *J. Biochem. (Tokyo)* 74, 155-160.
- McPherson, A. & Shlichta, P. (1988). Heterogeneous and epitaxial nucleation of protein crystals on mineral surfaces. *Science* 239, 385-387.
- McPherson, A. & Spencer, R. (1975). Preliminary structure analysis of canavalin from jack bean. *Arch. Biochem. Biophys.* 169, 650-661.
- Mirkov, T.E., Mathews, D.M., DuPlessis, D.H., & Dodds, J.A. (1989). Nucleotide sequence and translation of satellite tobacco mosaic virus RNA. *Virology* 170, 139-146.
- Ng, J.D., Ko, T.-P., & McPherson, A. (1992a). Cloning, sequencing, expression and crystallization of the vicilin protein, canavalin, from jack beans. *Plant Physiol.*, in press.
- Ng, J.D., Stinchcombe, T., Ko, T.-P., Alexander, E., & McPherson, A. (1992b). PCR cloning of the full length cDNA for the seed protein canavalin from the jack bean plant, *Canavalia ensiformis*. *Plant Mol. Biol.* 18, 147-149.
- Pusey, M.L. & Naumann, R. (1986). Growth kinetics of tetragonal lysozyme crystals. *J. Crystal Growth* 76, 593-599.
- Salemme, F.R. (1974). Preliminary crystallization data for cytochrome *c'* and *Rhodospseudomonas palustris*. *Arch. Biochem. Biophys.* 163, 423-425.
- Salemme, F.R. (1972). A free interface diffusion technique for the crystallization of proteins for X-ray crystallography. *Arch. Biochem. Biophys.* 151, 533.
- Smith, S.C., Johnson, S., Andrews, J., & McPherson, A. (1982). Biochemical characterization of canavalin: The major storage protein of jack bean. *Plant Physiol.* 70, 1199-1209.
- Sumner, J.B. & Howell, S.F. (1936). The isolation of a form of crystallizable jack bean globulin through the digestion of canavalin with trypsin. *J. Biol. Chem.* 113, 607-610.
- Valverde, R.A. & Dodds, J.A. (1986). Evidence for a satellite RNA associated naturally with the U5 strain and experimentally with the UI strain of tobacco mosaic virus. *J. Gen. Virol.* 67, 1875-1884.
- Valverde, R.A. & Dodds, J.A. (1987). Some properties of isometric virus particles which contain the satellite RNA of tobacco mosaic virus. *J. Gen. Virol.* 68, 965-972.
- Valverde, R.A., Heick, J.A., & Dodds, J.A. (1991). Interactions between satellite tobacco mosaic virus, helper tobamovirus, and their hosts. *Phytopathology* 81, 99-104.
- Weber, B.A. & Goodkin, P.E. (1970). A modified microdiffusion procedure for the growth of single protein crystals by concentration-gradient equilibrium dialysis. *Arch. Biochem. Biophys.* 141, 489-498.
- Wilson, A.J.C. (1949). The probability distribution of X-ray intensities. *Acta Crystallogr.* 2, 318-321.
- Wilson, A.J.C. (1970). *Elements of X-ray Crystallography*, Chapter 8. Addison-Wesley, Reading, Massachusetts.
- Wood, E.A. (1977). *Crystals and Light: An Introduction to Optical Crystallography*. Dover, New York.